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## Targeting the stromal microenvironment in chronic inflammation

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### Abstract

A characteristic feature of chronic inflammatory reactions is their persistence and predilection for certain sites. The molecular basis for such tissue tropism (as, for example, seen with metastatic spread) has until recently remained obscure, but recent studies have strongly implicated tissue-resident, stromal cells, such as macrophages, endothelial cells and fibroblasts. These cell types make attractive therapeutic targets as they help define the three-dimensional structure of tissues and are key orchestrators of the inflammatory infiltrate. Most current anti-inflammatory therapies target immune cells in an attempt to inhibit the production of pro-inflammatory mediators; however, an equally important target is the active induction of anti-inflammatory mediators involved in the resolution of inflammation. Recent work suggests that stromal cells are an important source of these mediators. Targeting of multiple signals may be required to inhibit tissue damage associated with inflammatory disease. Cells of the monocyte lineage are present as tissue-resident cells and interact closely with other stromal populations. These cells form an ideal target for modulation of the inflammatory environment as, in some cases, they appear to induce tissue repair. Therapeutic manipulation of the stromal microenvironment has been particularly effective in treating cancer and is likely to provide a novel method to achieve improved control of chronic inflammatory disease.

### Introduction

In the past ten years, a paradigm shift has occurred in the fields of inflammation and cancer cell research. Haemopoietic cells (see Glossary) are no longer seen and analysed in isolation, but need to be considered in the context of organ-specific stromal microenvironments. Such environments are composed of tissue-specific cells, such as fibroblasts, endothelial cells and resident macrophages, along with their highly specialised extracellular matrix (ECM) components. Evidence exists that tissue stromal cells are able to determine the type and duration of leucocyte infiltrates in an inflammatory response [1], whereas at the resolution of such responses, stromal cells also contribute to the withdrawal of survival signals and normalisation of chemokine gradients that allow infiltrating cells to undergo apoptosis or leave through draining lymphatics. Subversion of these pathways results in a switch to persistent inflammation, which remains remarkably stable [2•]. The relative lack of reagents that target the dynamic leucocyte–stromal interactions may account for the failure of current therapies to affect a permanent cure, as current treatments potentially miss many points where leucocyte–stromal interactions occur. By contrast, more recent therapies, including anti-tumour necrosis factor monoclonal antibodies and receptor Fc-fusion proteins, attempt to inhibit the complex cytokine networks between stromal and haemopoietic cells. In

targeting the stromal microenvironment, attempts are now being made to address the nature of the switch from resolving to persistent disease that underlies many chronic inflammatory diseases (Figure 1).

We consider a wider definition of stromal cells, to include tissue-resident cells encompassing those of the monocyte/macrophage lineage. These highly specialised cells form part of an organ-specific stromal network. As evidence of plasticity between haemopoietic and mesenchymal lineages accumulates, it is clear that neither conventional mesenchymal nor tissue-resident haemopoietic cells can be considered in isolation. We consider the recent evidence for involvement of stromal cells and tissue macrophages in rheumatoid arthritis (RA) and chronic liver disease, explore the relationship between inflammation, wound healing and cancer, and discuss the pharmacological targets currently or likely to be pursued in the next few years.

## What is a stromal cell?

Two broad historical definitions of stromal cells exist. The first is that they are cells of mesenchymal origin that are non-epithelium, non-endothelium and non-haematopoietic. The second is that they are the cells resident within the tissue stroma and therefore include fibroblasts, endothelial cells and tissue-resident leucocytes. Irrespective of these definitions, stromal cells are responsible for defining the specialised architectures of organs and tissues through the secretion of ECM components and characteristic cytokine and chemokine combinations. Stromal cells have been defined in terms of their embryological origins and lineage relationships, and are generally considered to be mesenchymal in origin. However, the embryological mesenchyme, from which fibroblasts are derived, is not in itself a germ layer (usually defined as ectoderm, endoderm and mesoderm), but is variably considered to be either wholly composed of embryonic mesoderm or a combination of the mesoderm and ectoderm or endoderm layers. For instance, in the head and neck, some mesenchyme is derived from neural crest cells (and hence from ectoderm). Unfortunately the blood cell lineages are also derived from the mesoderm, which means that haemopoietic stem cells are also technically mesenchymal stem cells. Cell populations have now been identified that appear to blur the distinction between haemopoietic and non-haemopoietic populations, including the fibrocyte [3]. Moreover, other unexpected shifts in lineage have been reported, including differentiation from neural stem cells into myeloid and lymphoid haemopoietic lineages [4]. The standard approach to cellular classification is, therefore, becoming increasingly awkward and means that some of the therapeutic effects of bone marrow transplantation need to take these issues into account. The crucial importance of stromal populations in the regulation of specialised tissue function and architecture is demonstrated by the strong association of stromal cell defects with tumourigenesis [5,6].

Our inability to precisely define the prototype stromal cell, the fibroblast, using phenotypic markers has significantly retarded progress in the field. Descriptions of different fibroblasts, therefore, generally rely upon a combination of morphology, phenotypic markers (often negative, ruling out other contaminants) and functional features, whereas immunohistochemistry and *in situ* hybridisation often rely almost entirely upon morphological features. Consequently, the discrete targeting of stromal cells such as fibroblasts has been fraught with difficulty. However, transcriptional profiling is now changing this situation; Chang *et al.* [7] have convincingly demonstrated that fibroblasts from different sites can be consistently separated by their transcriptomes, which offers the possibility of improved characterisation.

## Stromal cells and inflammation

### Fibroblasts

The most abundant cells of the stroma are fibroblasts, which are responsible for the synthesis and remodelling of EMCs. In addition, their ability to produce and respond to growth factors allows reciprocal interactions with other stromal cell types and with adjacent epithelial and endothelial structures. As a consequence, fibroblasts play a critical role during tissue development and homeostasis and are often described as having a 'sentinel' or 'landscaping' function. Moreover, these functions contribute to the pathology of many diseases either directly, for example by overproduction of matrix components during fibrosis, and/or indirectly, for example by influencing the behaviour of neighbouring cell types.

Rheumatoid arthritis synovial fibroblasts (RA SFbs) provide a convincing example of how stromal cells contribute to the persistence of inflammation. RA SFbs are now known to be direct effectors of tissue injury and remodelling. They display an imprinted phenotype that is stable under *in vitro* culture conditions, and which extends to functionally important outcomes, such as cartilage invasion, as demonstrated in severe combined immunodeficient (SCID) mouse models [8]. RA SFb-mediated erosion of cartilage and bone determines disease outcome for the majority of RA patients [9]. Furthermore, evidence suggests that, through secretion of cytokines and chemokines, SFbs play a role in the persistence of inflammation in the synovium through recruitment and retention of effector cells of the immune system [10].

Type I interferons are produced by the expanded stromal population of SFbs and macrophages, which results in a lack of proliferation but also a block of the apoptotic signals that normally result in a coordinated wave of T lymphocyte death at the conclusion of an inflammatory response [11-13]. Unexpectedly, CD45RO<sup>+</sup> T lymphocytes were found to express CXCR4 receptors at high levels in the rheumatoid synovium. The ligand for CXCR4, CXCL12, is highly expressed on endothelial cells at the sites of T cell accumulation [13-15]. In addition, stromal-cell-derived tumour growth factor- $\beta$  (TGF- $\beta$ ) is responsible for the upregulation of CXCR4 receptors on T cells in the synovium, and may be responsible for the production of a new subset of interleukin (IL)-17-producing cells called T<sub>H</sub>-17 that have been strongly implicated in immune-mediated inflammatory diseases [16••]. Cross-talk between chemokine and cytokine networks may operate to reinforce the retention of T cells by CXCL12, as locally raised IL-1 and tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) levels cause synovial fibroblasts and macrophages to secrete IL-15, which upregulates CXCR4 on T cells, and may thus also contribute to the retention of T lymphocytes [14]. There is, therefore, clear evidence in support of the hypothesis that aberrant expression of constitutive chemokines by SFbs is responsible for retention of T cells within the RA synovium. These studies have been supported by data on the targeting of CXCR4 in murine models of arthritis where the CXCR4 antagonists AMD3100 and 4F-benzoyl-TN14003 ameliorated disease severity in DBA/1 (interferon- $\gamma$  receptor deficient) mice and collagen-induced arthritis (CIA), respectively [17,18]. It is possible that CXCR4 antagonists will be of use in the therapy of RA provided that toxicity issues caused by stem cell mobilisation from the bone marrow, which depend upon CXCL12/CXCR4 interactions, do not pose a major problem.

The unique, imprinted phenotype of RA SFbs bears remarkable phenotypic similarities to stromal cells of the bone marrow concerned with the recruitment and support of haemopoietic cells [19]. Recent studies have suggested that the phenotype of RA SFb is accounted for by accumulation of blood-borne stromal progenitor cells (mesenchymal progenitor cells) [19]. Other possible sources of stromal cells in inflammatory diseases

include epithelial to mesenchymal transition, a phenomenon observed in inflammatory diseases of the kidney at sites of epithelial injury [20]. Targeting of such transdifferentiation processes may prove useful in retarding fibrotic diseases such as systemic sclerosis [21].

### Endothelial cells

Endothelial cells are responsible for vascular tone, haemostasis and the transport of cells and soluble mediators throughout the body, with diverse phenotypes ranging from large arteries to capillaries and lymphatic endothelium. Tissue injury results in release of lipid mediators, endothelial upregulation of adhesion molecules, and production and release of chemokines, such as CXCL8 (IL-8), which results in adherence and transmigration of leucocytes. Recent advances have improved our understanding of the transcriptional diversity of endothelial cells [22], and also of the interactions that occur between endothelial cells and associated stromal cells, such as pericytes, macrophages and fibroblasts [23,24]. Growth of inflammatory or neoplastic tissue requires new blood vessel formation or angiogenesis to occur. Angiogenesis itself requires the presence of new organ-specific vascular cells, a network of associated supporting stromal cells (pericytes) and ECM, which are normally responsible for tight control through factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [25]. Studies targeting VEGF are compelling. They demonstrate that blockade of VEGF, its receptors or its downstream signalling pathways is able to ameliorate disease in both *ex vivo* and *in vivo* models. This suggests that VEGF activity is necessary for the persistence of inflammation in arthritis. A VEGF receptor–Fc construct suppressed synovial endothelial proliferation in RA synovial tissue explants [26]. In CIA models, anti-VEGF has been shown to reduce arthritis onset and severity, whereas VEGF receptor Flt-1 blockade reduces synovial angiogenesis [27]. Antibodies to VEGF-R1 and a VEGF-R1 tyrosine kinase inhibitor in K/BxN murine arthritis also led to amelioration of disease [28]. It is likely that targeting of angiogenesis will be a therapeutic reality in the near future.

A recent breakthrough in the elucidation of trafficking pathways has been the development of specific markers for lymphatic, as opposed to vascular, endothelium. Of these, the most specific is LYVE-1: a hyaluronan receptor expressed exclusively on draining lymphatic vessels [29]. Markers such as LYVE-1 now allow the potential targeting of lymphatic vessels, which control trafficking out of inflammatory environments. In RA synovium, deregulation of chemokine gradients occurs, which suggests that tissue expression of constitutive chemokines plays a role in lymphocyte retention by subverting the normal CK gradient (which causes egress through lymphatics) towards draining lymph nodes [30].

### Monocytes as haemopoietic/stromal cell intermediates

Upon stimulation, monocytes differentiate into a wide variety of cells, including macrophages, dendritic cells and osteoclasts [31,32]; however, emerging data suggest that monocytes may be able to differentiate down a number of stromal cell lineages to produce endothelial cells and fibroblasts [33]. The presence of such stromal plasticity in the monocyte lineage may help to address the wider question of the haematopoietic origins of stromal cells seen in inflammation and wound healing.

Fibrocytes are adherent cells with a spindle-shaped morphology arising from within the CD14-positive (monocyte) fraction of peripheral blood, and they express major histocompatibility complex class II as well as type I collagen and other ECM molecules [34]. They have been shown to rapidly enter sites of tissue injury [35] where they elaborate collagen-based matrix and have been proposed to differentiate along fibroblast lineages under the influence of cytokines, particularly TGF- $\beta$  [3]. Fibrocytes have also been shown to contribute to tissue remodelling in models of inflammatory lung disease [36]. In lung

fibrosis models, the CXCL12–CXCR4 chemokine–receptor pair has been identified as essential in the recruitment of fibrocytes because targeting of CXCR4 with monoclonal antibodies was shown to ameliorate disease [37••].

Further research linking monocytes and tissue resident stromal cells involves CX3CR1 — the receptor for the chemokine fractalkine. Peripheral blood monocytes can be split into two populations depending on their expression of this receptor. The CX3CR1-low population (‘inflammatory monocytes’) are recruited to inflammatory sites, whereas the CX3CR1-high population (‘immune monocytes’) are recruited to tissues where they differentiate into various resident cell populations, including Kupffer cells and Langerhans cells [38]. It may, therefore, be possible to selectively target those cells that participate in persistent inflammatory responses without impairing physiological immune function.

### Selective depletion of stromal cell subsets

The proven therapeutic effects of antibodies that deplete specific subsets of cells in the treatment of leukaemia and transplant rejection has provided a proof-of-concept that such cell-depletion strategies can be very effective. Is it possible that persistent inflammation could be ameliorated by targeted depletion of specific cell subpopulations, such as monocytes, macrophages and fibroblasts? Data from models of hepatic fibrosis suggest that resident populations of hepatic macrophages are able to modulate the processes of fibrotic progression and subsequent liver recovery. Stellate cells (specialised liver fibroblasts) are known to differentiate during the process of hepatic injury and fibrosis towards a pro-fibrotic, secretory myofibroblast phenotype under the influence of cytokines such as TGF- $\beta$ 1, of which hepatic macrophages are a potentially important source [39]. High expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) by stellate cells/myofibroblasts appears to maintain their survival and inhibit degradation of matrix [40]. During recovery from fibrosis, myofibroblasts undergo apoptosis accompanied by a fall in TIMP-1 expression and degradation of scarring matrix [41]. Expression by macrophages of TNF-related apoptosis-inducing ligand (TRAIL) by macrophages is one mechanism by which stellate cell apoptosis has been proposed to occur [42]. Using a transgenic mouse model of fibrosis, which allowed discrete depletion of macrophage lineage cells under the influence of diphtheria toxin, Duffield *et al.* [43••] were able to demonstrate a fascinating divergence of macrophage functional regulation of these processes. Depletion of macrophages during the progression phase of fibrosis resulted in decreased scarring and fewer myofibroblasts; however, when macrophages were depleted in the early phases of recovery, sustained accumulation of scarring matrix occurred [43••] (Figure 2).

Divergence of macrophage phenotypes during opposing processes of injury and repair could be due either to phenotypic switching of existing cells or to recruitment of new, functionally different, cells: a question that has yet to be resolved [39]. However, although depletion of fibroblast subsets remains a distant possibility, macrophage lineage tissue-resident cells may be a viable target for depletion, allowing us to modify interactions between different stromal cell subpopulations with therapeutic benefit.

### Relationship between inflammation and repair: lessons from targeting stromal cells in cancer

Wound healing, chronic persistent inflammation and cancer exhibit significant parallels. They are all associated with deposition of new ECM and expansion of adjacent stromal cells, including fibroblasts, smooth muscle cells and endothelial cells. Furthermore, many authors have drawn parallels between tumourigenesis and wound healing: classically describing tumours as wounds that fail to heal [44]. Chang *et al.* [45••] recently



demonstrated that the transcriptional response programme of fibroblasts to serum, as seen in wound healing, was also seen in tumour and associated stromal cells. The presence of its signature in tumour samples was found to be predictive of metastasis and death in common cancers. Much has been learnt about the communication between networks of stromal cells within tissues — pathological alterations of which are crucial to the process of tumourigenesis. Parrinello *et al.* [46] found that senescent fibroblasts were able to disrupt the normal branching morphogenesis of breast epithelial cells by secreted factors, including matrix metalloproteinases (MMPs) and epithelial growth factors. When cultured with premalignant cells, this disruption resulted in loss of differentiation representing malignant transformation. This bystander effect of senescence, itself a means of controlling potentially neoplastic cells, appears paradoxical and has been suggested to account for the age-related incidence of epithelial cancers. It is also an indication that simply inducing apoptosis or senescence of putative inflammatory stromal cells may have adverse effects. By contrast, most researchers describe an activated, secretory phenotype of tumour-associated fibroblastic cells [5,6]. A number of important cytokines and growth factors has been described that contribute to neoplastic transformation of epithelial cells by activated tumour-associated fibroblasts: these include hepatocyte growth factor, TGF- $\beta$ , MMPs and FGFs. Crucially, in contrast to senescent fibroblastic cells, tumour-activated fibroblasts appear able to transform normal, in addition to pre-malignant, epithelial cells [6]. Blockade of both TGF- $\beta$  and the hepatocyte growth factor receptor c-Met has been effective in inhibiting both the development and the metastasis of neoplasms in animal models [47,48]. Conversely, and confirming the essential organ-like nature of the tumour as both ‘cancer cells’ and their surrounding stroma, human breast cancer xenografts are unable to implant successfully into mice unless accompanied by human tumour-derived fibroblasts [49••].

Release of growth factors, such as TGF- $\beta$ , bFGF, VEGF, platelet-derived growth factor, MMPs and proteolytic enzymes, by tumour cells in turn modifies the tumour stroma and its cells to become angiogenic and pro-migratory [5]. The angiogenic properties of tumours are therefore a cooperative product of tumour cells, tumour-associated stromal cells and angiogenic factors released by proteolysis of the ECM. Studies using a skin surface model of tumourigenesis indicated that expression of VEGF was ubiquitous amongst benign and malignant tumours; however, it was the upregulation by stromal endothelial cells of VEGF receptors that led to tumour invasion, with blockade of such receptors halting tumour progression [50]. The concept of tumour stroma ‘normal-isation’ has now become an accepted aspect of new oncology therapies. Clinical studies of angiogenesis inhibitors and antibodies against ECM components, such as tenascin, have been favourable, and inhibitors of MMPs, overexpression of TIMPs and blockade of integrin signalling have all shown promise in pre-clinical trials [5]. Results of studies examining the interactions between endothelial cells and their associated pericytes underline the importance of targeting the stroma as a whole. Bergers *et al.* [51] have shown that endothelial cells release PDGF, which induces VEGF production from pericytes leading to bidirectional conversations between the two cell types. Interrupting these conversations by using PDGF inhibitors proved to be more effective therapy than using VEGF inhibitors alone. Interestingly, although VEGF inhibitors lost their inhibitory effect in later stage tumours, targeting of the pericytes helped even late stage tumours to regress. The authors have subsequently shown that pericyte precursors are partly recruited from the bone marrow to tumour perivascular sites [52•]. Recent evidence using breast carcinoma models have shown that tumour-associated fibroblasts secrete CXCL12, which results in increased promotion of carcinoma cell growth compared with control fibroblasts, but also to recruitment of endothelial cell precursors [53••].

## Conclusions

Stromal cells, such as endothelium, fibroblasts and tissue-resident macrophages, have great potential as therapeutic targets. They are responsible for orchestrating and maintaining the presence of inflammatory infiltrates. Classical approaches using antibody blockade of cytokines such as TNF $\alpha$  have been spectacularly successful. The targeted depletion of tissue-resident cells using cell depletion opens an alternative and exciting new avenue. Furthermore, experience in the field of cancer medicine has highlighted approaches using unconventional agents, such as protease inhibitors and matrix components, and emphasised the necessity to understand the conversations occurring between different stromal cell populations. Lastly, naturally occurring stromally derived factors that aid the resolution of inflammation have great immediate potential for therapeutic use and are likely to become important targets for further research.

## Glossary

<b>Angiogenesis</b>	the growth of new blood vessels
<b>Haemopoietic cells</b>	those cells considered developmentally to arise from haemopoietic stem cells, principally the erythroid, lymphoid and myeloid lineages
<b>Extracellular matrix</b>	the complex network of polysaccharides and proteins secreted by cells, forming a structural component of tissues that also influences their development and physiology
<b>Tissue-resident cells</b>	cells characteristic of, and remaining within, a given tissue regardless of origin. Includes fibroblasts, endothelial cells, pericytes and tissue-specific macrophages
<b>Tumourigenesis</b>	the process by which tumours are formed

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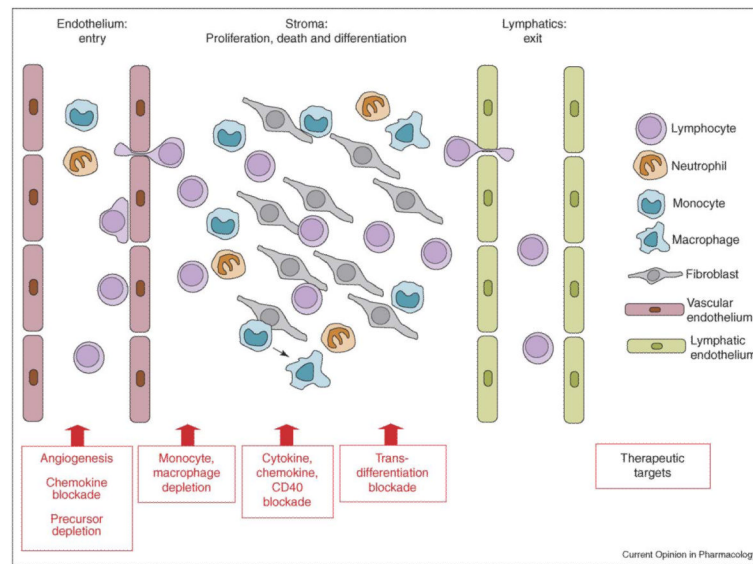
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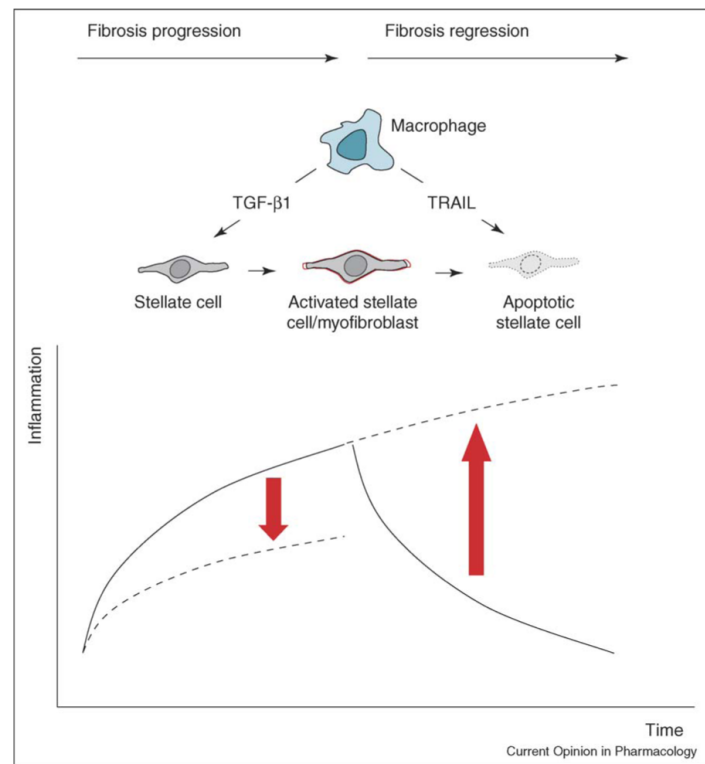
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**Figure 1.**

Leukocyte–stromal interactions in chronic inflammatory disease. The molecular basis by which leukocytes leave the circulation and migrate across endothelium has been well studied; stromal and lymphatic trafficking remain less well understood. Leukocytes captured by selectin/ligand interactions roll, sampling the presence of chemokines and other activation markers on the endothelium and associated matrix proteins. Infiltrating cells undergo firm adhesion then migrate through the endothelial layer following chemokine gradients into the tissue stroma. Possible therapeutic targets here include inhibition of angiogenesis using VEGF blockade, and blockade of specific chemokines and their receptors including CXCL12/CXCR4. Alternatively, depletion of precursor cell populations such as fibrocytes and endothelial precursors offers a means to control stromally mediated inflammation and angiogenesis. Within the stroma, some cells are destined to die, such as neutrophils. Others such as monocytes can differentiate into cells destined to die, such as macrophages; others might proliferate. Potential targets within the stroma include deletion of specific monocyte/macrophage populations with a pathological role, as suggested by work in models of liver fibrosis (see text). Blockade of novel cytokines and chemokines, and also of stromal/leukocyte cell-mediated interactions via molecules such as CD40, have immediate therapeutic potential. Also potentially important is blockade of the transdifferentiation of tissue resident cells such as monocytes into pathologically important macrophage populations, or their accelerated apoptosis. Those cells destined to recirculate must follow other chemokine gradients towards the lymphatic endothelium and exit from the tissue towards draining lymph nodes. The endothelium regulates entry; the stroma regulates proliferation, survival and differentiation; and the lymphatics regulate exit.



**Figure 2.**

Divergence of macrophage function in fibrotic liver disease. During fibrosis progression (rising solid line on graph), TGF-β1 is a potential macrophage-derived stimulator of stellate cell activation. Depletion of macrophages during this phase results in decreased scarring and fewer myofibroblasts, with decreased inflammation (small red arrow). During fibrosis regression (falling solid line on graph), stellate cell apoptosis can occur under the influence of macrophage-derived TRAIL. Depletion of macrophages early during fibrosis regression results in sustained inflammation and accumulation of scarring matrix (large red arrow). Hepatic macrophages thus have divergent effects on liver stellate cell activation in models of fibrosis.